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Award Number: DAMD17-98-1-8357

TITLE: Mechanisms through which Rat Mammary Gland  
Carcinogenesis is Preferentially Initiated by H-Ras over K-Ras  
Signaling Pathways

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REPORT DATE: July 2001

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command  
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;  
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20040524 124

**REPORT DOCUMENTATION PAGE**Form Approved  
OMB No. 074-0188

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<b>1. AGENCY USE ONLY (Leave blank)</b>		<b>2. REPORT DATE</b> July 2001	<b>3. REPORT TYPE AND DATES COVERED</b> Annual Summary (1 Jul 98 - 30 Jun 01)	
<b>4. TITLE AND SUBTITLE</b> Mechanisms through which Rat Mammary Gland Carcinogenesis is Preferentially Initiated by H-Ras over K-Ras Signaling Pathways			<b>5. FUNDING NUMBERS</b> DAMD17-98-1-8357	
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<b>9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)</b> U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012			<b>10. SPONSORING / MONITORING AGENCY REPORT NUMBER</b>	
<b>11. SUPPLEMENTARY NOTES</b>				
<b>12a. DISTRIBUTION / AVAILABILITY STATEMENT</b> Approved for Public Release; Distribution Unlimited				<b>12b. DISTRIBUTION CODE</b>
<b>13. ABSTRACT (Maximum 200 Words)</b>  This research distinguishes mechanisms through which activated Ras induces rat mammary gland carcinogenesis. Experiments expressing H-Ras and K-Ras chimeric proteins in mammary gland (Aim 1A) investigate hypothesis one: greater frequency of H-Ras carcinogenesis than K-Ras carcinogenesis in rat mammary gland results from differences in the last 20 amino acids of H-Ras and K-Ras. K-Ras with an H-Ras c-terminus is as tumorigenic as H-Ras, supporting hypothesis one: however, H-Ras with a K-Ras c-terminus is also as tumorigenic as H-Ras, suggesting a unique characteristic of intact K-Ras limits mammary carcinogenesis. Expression of Raf-Caax (Aim 1B), does not induce tumors, suggesting hypothesis two: individual Ras effectors won't initiate rat mammary carcinogenesis, rather multiple effectors must act synergistically. However, each Ras effector loop mutant (G37-Ras activates RalGDS, E38-Ras activates Raf, and C40-Ras activates PI3K) (Aim 2A), individually induces mammary carcinomas. Carcinoma induction from n-terminus truncated Raf ( $\Delta$ Raf) confirms Raf activation alone will induce carcinomas, eliminating hypothesis two. To investigate the apparent inconsistency that E38-Ras and $\Delta$ Raf cause tumors but Raf-Caax does not, these Raf mutations were combined ( $\Delta$ Raf-Caax). Since $\Delta$ Raf-Caax constructs also fail to induce mammary carcinomas, we conclude that c-terminal lipid modification of Raf is not oncogenic in rat mammary gland.				
<b>14. SUBJECT TERMS</b> Breast Cancer			<b>15. NUMBER OF PAGES</b> 16	
			<b>16. PRICE CODE</b>	
<b>17. SECURITY CLASSIFICATION OF REPORT</b> Unclassified	<b>18. SECURITY CLASSIFICATION OF THIS PAGE</b> Unclassified	<b>19. SECURITY CLASSIFICATION OF ABSTRACT</b> Unclassified	<b>20. LIMITATION OF ABSTRACT</b> Unlimited	

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#### Introduction:

This research distinguishes mechanisms through which activated Ras induces rat mammary gland carcinogenesis. The primary experimental model relies on production of very high titer, replication-incompetent, retroviral expression vectors. These retroviral vectors are infused into the central duct of rat mammary gland to confer expression of experimental proteins to endogenous mammary epithelial cells *in situ*. Aim 1 of this research focuses on differences between H-Ras and K-Ras proteins, while Aim 2 focuses on different Ras effector pathways. *In situ* expression of activated H-Ras is 5-10 times more tumorigenic than K-Ras in rat mammary gland. These experiments expressing H-Ras and K-Ras chimera proteins in rat mammary gland suggest carcinogenic limitations unique to intact K-Ras proteins. Activated Ras proteins bind other signal transduction proteins, such as Raf, PI3K, and RalGDS. These *in situ* expression experiments with Ras effector loop mutants (ELM's), find activated Ras efficiently induces mammary carcinomas through any one of three individual Ras effector pathways, though Raf was clearly the dominant pathway. Furthermore we find n-terminal truncated Raf ( $\Delta$ -Raf) induces carcinomas. However, Raf-Ras fusion proteins (Raf-H-Caax or Raf-K-Caax) and  $\Delta$ -Raf-Ras fusion proteins ( $\Delta$ -Raf-H-Caax or  $\Delta$ -Raf-K-Caax) fail to induce carcinomas. These results have important implications for targeted treatment of different tumor types with novel chemotherapeutic agents.

Body:

Aim 1 is to distinguish which H-Ras and K-Ras protein domains result in different potential to transform *in situ* mammary epithelial cells. The hypothesis of Aim 1 is; the different potential of H-Ras and K-Ras to initiate rat mammary gland carcinogenesis, results from differences in the last 20 amino acids of H-Ras and K-Ras. This hypothesis is being tested with expression of chimeric Ras proteins.

Aim 1A is comparing differences in rat mammary tumor formation resulting from expression of activated H-Ras and K-Ras with their carboxyl ends exchanged. Expressing K-Ras with an H-Ras c-terminus seems as tumorigenic as H-Ras, supporting the hypothesis of Aim 1: however, H-Ras with an K-Ras c-terminus also seems as tumorigenic as H-Ras, suggesting a unique characteristic of intact K-Ras limits carcinogenesis in mammary gland.

Aim 1B was to compare differences in rat mammary tumor formation resulting from expression of Raf activated by fusion to the carboxyl end of H-Ras (Raf-H-Caax) to Raf activated by fusion to the carboxyl end of K-Ras (Raf-K-Caax). The results of Aim 1B don't reflect on the hypothesis of Aim 1, since neither form of Raf resulted in a single tumor. The results of Aim 1B suggest the hypothesis of Aim 2: no individual Ras effector will initiate transformation, multiple effectors must synergise.

New antibodies and fractionation techniques that allow staining for individual Ras family members and fractionation of plasma membrane micro domains make the methods proposed for Aim 1C antiquated.

Aim 2 is to distinguish the pathway(s) critical to transformation of *in situ* mammary epithelial cells by activated H-Ras. As mentioned earlier, the results of Aim 1B suggest the hypothesis of Aim 2: no individual Ras effector will initiate transformation, multiple effectors must synergise. Raf is thought to be the most tumorigenic of Ras effectors, so lack of tumor formation from expression of Raf-Caax, suggests Raf must synergise with an additional Ras effector(s). The results from Aim 1B contrast with those from Aim 2A.

Aim 2A was expressing individual Ras effector loop mutants (ELM's), which target particular Ras effectors (G37-Ras activates RalGDS, E38-Ras activates Raf, and C40-Ras activates PI3K). The results of Aim 2A experiments substantially weaken the hypothesis of Aim 2. Each of the Ras ELM's causes tumors individually. Tumors from expressing G37 or C40 Ras had a long latency (11-12 weeks), similar latency to tumors from expression of non-activated wt-H-Ras (15 weeks). E38-Ras, which activates Raf, is very tumorigenic with a short latency (3-5 weeks), as seen with activated H-Ras. This suggests Raf is the critical tumor generating effector of activated H-Ras. The coexpression experiments proposed for Aims 2B and 2C are no longer relevant due to the results of Aim 2A.

The contrasting results from Aim 1B and Aim 2A suggest Raf-Caax is defective for *in situ* tumor formation, or (and) E38-Ras is activating an additional effector pathway(s). RT-PCR of mRNA from Raf-H-Caax or Raf-K-Caax infused glands, shows some cells in the gland are still expressing the RNA months after infusion. Transformation of cultured cells by the Raf-Caax vectors, and immunostaining of infected cells has confirmed expression of the Raf proteins from the vectors. The first three Raf proteins expressed carried epitope tags for future identification (tag-wt-Raf, tag-Raf-H-Caax, and tag-Raf-K-Caax). To rule out an immunoresponse to the protein tag, Raf expression vectors were reconstructed without tags (wt-Raf, Raf-H-Caax, and Raf-K-Caax). A-terminus truncation of Raf is another way to increase its kinase activity. To further investigate the carcinogenic potential of Raf, a-terminus truncations were also constructed ( $\Delta$ Raf,  $\Delta$ Raf-H-Caax, and  $\Delta$ Raf-K-Caax). Of the nine Raf vectors tested, only  $\Delta$ Raf causes tumors.

Formation of rat mammary tumors from expression of Ras-ELM's weakened hypothesis two, and tumor formation from  $\Delta$ Raf eliminates hypothesis two.  $\Delta$ Raf and E38-Ras results showing Raf activation can induce carcinomas seem inconsistent with Raf-Caax results. Further investigation combining both activating mutations (amino terminal truncation and membrane localization motifs) in one Raf construct ( $\Delta$ -Raf-Caax) suggests membrane targeting of activated Raf can actually block tumorigenesis. These results have important implications for targeted treatment of different tumor types with novel chemotherapeutic agents.

Key research accomplishments:

- Designed, constructed, produced, concentrated, titered, and tested for absence of helper virus in 17 new replication-defective retroviral expression vectors (all of these could be useful researching other models).
  - Four H&K-Ras domain swap vectors: JR-H-Ras-H-Caax, JR-H-Ras-K-Caax, JR-K-Ras-K-Caax, and JR-K-Ras-H-Caax.
  - Four Ras effector loop mutant vectors: JR-V12-H-Ras, JR-G37-V12-H-Ras, JR-E38-V12-H-Ras, and JR-C40-V12-H-Ras.
  - Three tagged Raf vectors: JR-tag-Raf-H-Caax, JR-tag-Raf-K-Caax, and JR-tag-wt-Raf.
  - Three Raf vectors: JR-Raf-H-Caax, JR-Raf-K-Caax, and JR-wt-Raf.
  - Three n-terminal truncated Raf vectors: JR- $\Delta$ Raf-H-Caax, JR- $\Delta$ Raf-K-Caax, and JR- $\Delta$ Raf
- Found unique characteristics of intact K-Ras limit carcinoma induction in rat mammary gland.
  - Both H&K-Ras chimeras are strongly tumorigenic, like H-Ras.
- Found membrane targeting of Raf alone is not able to transform mammary epithelial cells *in situ*.
  - Rats infused with tagged Raf vectors and Raf vectors do not develop carcinomas.
  - RT-PCR on RNA from tag-Raf-Caax infused glands to confirm expression of tag-Raf-Caax RNA's in infused mammary glands.
  - Immunostaining to confirm expression of tag-Raf-Caax proteins in infected cultured cells.
- Found activated Ras efficiently induces mammary carcinomas through any one of three individual Ras effector pathways (Raf, PI3K, or RalGDS), though Raf is clearly dominant.
- Found Raf activated by Ras (without PI3K or RalGDS) induces mammary carcinomas as rapidly as activated Ras (4 weeks).
- Found PI3K or RalGDS activated by Ras induce mammary carcinomas almost as slowly (11 and 12 weeks respectively) as non-activated wt-H-Ras (15 weeks).
- Found  $\Delta$ Raf induces mammary carcinomas almost as quickly (6 weeks) as activated Ras.
- Found the combination of both Raf activating mutations (amino terminal truncation and membrane localization motifs) in one Raf construct ( $\Delta$ -Raf-Caax) does not induce mammary carcinomas. Lipid modification on the c-terminus of  $\Delta$ -Raf somehow prevents tumorigenesis.

## REPORTABLE OUTCOMES:

McFarlin, D. R. (2002) Characterizing oncogenic Ras effectors in a rat mammary gland model. Dissertation submitted/published in partial fulfillment of the requirements for the degree of Doctor of Philosophy (Cellular and Molecular Biology) at the University of Wisconsin—Madison. Ph.D. awarded August 2002.

McFarlin, D. R. and Gould, M. N. (2003) Rat mammary carcinogenesis induced by in situ expression of constitutive Raf kinase activity is prevented by tethering Raf to the plasma membrane. *Carcinogenesis*. In press

McFarlin, D. R., Lindstrom M. J., Gould M. N. (2003) Affinity with Raf is sufficient for Ras to efficiently induce rat mammary carcinomas. *Carcinogenesis*. Jan;24(1):99-105.

\*Kim K, Lindstrom MJ, Gould MN (2002). Regions of H- and K-ras that provide organ specificity/potency in mammary cancer induction. *Cancer Res*. Mar 1;62(5):1241-5.

\*My (Daniel R. McFarlin's) efforts (several months) and The U.S. Army Medical Research and Materiel Command under DAMD17-98-1-8357 contributed significantly to development of this work. My efforts (while funded by The U.S. Army Medical Research and Materiel Command under DAMD17-98-1-8357) include construction, production, concentration and infusion of four H-Ras and K-Ras chimeric vectors, as well as rat mammary palpation, necropsies and other follow up. The vectors constructed and infused and the data resulting from my efforts were used for construction of additional vectors that were the foundation of this paper.

McFarlin, D.R., Kennan, W.S. and Gould M.N., "Ras signaling through Raf is more tumorigenic in rat mammary gland than Ras signaling through PI3K or RalGDS." Abstract #2475 in *Proceedings of the American Association for Cancer Research*, vol.40, p.374, 1999.  
Presented as a poster at the 90<sup>th</sup> annual meeting of the American Association for Cancer Research in April of 1999.  
A poster with the same title and authors was also presented at the "Genetics, Genomes, and Molecules" symposium on the University of Wisconsin—Madison campus in May of 1999.

The U.S. Army Medical Research and Materiel Command under DAMD17-98-1-8357 supported this work

## Conclusions:

- 1) Unique characteristics of intact K-Ras protein limit carcinoma induction in rat mammary gland.  
--Further investigation should include individual H-Ras amino acid substitutions in a K-Ras backbone to determine which are required for limiting carcinogenesis. Combined with yeast two hybrids using those constructs and protein modeling with the known crystal structures of Ras, the proteins and mechanisms responsible for limiting K-Ras carcinogenesis may be determined. This knowledge is likely to provide novel targets for treatment of human tumors with activating mutations in K-Ras, the most frequently mutated Ras family member in human cancer.
- 2) Fusion of Ras membrane localization signals to the c-terminus of Raf is not sufficient for inducing rat mammary carcinomas.  
--Taken in isolation, this result misleads one to false conclusions about the sufficiency of Raf to induce mammary carcinogenesis. However when combined with E38-V12-Ras and  $\Delta$ -Raf results, it creates important questions about interpretations in current literature regarding the weakness of Raf in carcinogenesis, which are based on lack of transformation from Raf-Caax.
- 3) Activated Ras efficiently induces mammary carcinomas through any one of three individual Ras effector pathways, though Raf is clearly the dominant pathway.  
--All three pathways should be studied further, each could provide novel targets useful for treatment of some tumors. Further study should include gene chip comparisons of mRNA expression patterns in these tumors, and possibly *in situ* expression of activated PI3K and RalGDS proteins.
- 4) PI3K or RalGDS activated by Ras induce mammary carcinomas almost as slowly (11 and 12 weeks respectively) as non-activated wt-H-Ras (15 weeks).  
--*In situ* expression of activated PI3K and RalGDS proteins should be used to investigate whether C40-Ras and G37-Ras carcinoma induction results from activation of PI3K and RalGDS or from deregulated over-expression H-Ras protein.  
--Similar latency to tumorigenesis suggests that there may be similar mechanisms involved. C40-Ras, G37-Ras, and wt-H-Ras all retain affinity for RasGAP. Deregulated over-expression of Ras could sequester RasGAP causing an increase in endogenous Ras activity. C40-Ras and G37-Ras may be better at sequestering RasGAP because they cannot be deactivated, so affinity with RasGAP is not lost from the interaction, as would happen with wt-H-Ras. The more transient interaction with wt-H-Ras would require higher levels of Ras protein or greater amounts of background activation (prolactin, insulin etc.) to overwhelm the same amount of RasGap. Greater amounts of background activation may be provided by the rat aging process, which could explain the longer latency to wt-H-Ras tumor induction.  
--Expression of R34-Ras, which lacks affinity with RasGAP would begin to investigate this possibility. Combining the R34 mutation with the other individual Ras effector loop mutations used in the current study would further test this hypothesis. However yeast two hybrids would need to be used to determine if combining mutations resulted in the expected protein binding patterns, a debatable likelihood.



- 5) Raf activated by Ras (without PI3K or RalGDS) induces mammary carcinomas as rapidly as activated Ras (4 weeks).
  - Raf is the dominant oncogenic Ras signaling pathway, hence this pathway and its interaction with Ras became, and should continue to be, the preferential focus of further investigation in the gland.
  - Activation of the Ras oncogene is one of the most common molecular events in the etiology of human cancer, making Ras an important target for the prevention and treatment of cancer. However, Ras is also involved as a master switch in many normal physiologic functions. Unfortunately, this increases the likelihood that inhibiting Ras will lead to systemic toxicity. Targeting the Ras pathway that is key to oncogenesis (Raf) may minimize drug toxicity and thus improve therapeutic index.
- 6)  $\Delta$ Raf induces mammary carcinomas almost as quickly (6 weeks) as activated Ras.
  - The clear oncogenic potency of Raf in rat mammary gland warrants further investigation. *Raf* mutations have not been commonly found in human tumors, but this may result from a lack of effective screening. Activating point mutations to *Raf* have been found in mouse tumors that lack *Ras* mutation. Since human breast cancers lack *Ras* mutations, screening them for *Raf* mutation is suggested. For reasons too detailed to go into here, I suspect estrogen receptor negative and *neu* negative mammary tumors (which currently have the fewest treatment options) are most likely to possess activating point mutations in *Raf*.
- 7) The combination of both Raf activating mutations (amino terminal truncation and membrane localization motifs) in one Raf construct ( $\Delta$ -Raf-Caax) does not induce mammary carcinomas.
  - This first step investigating the mechanisms involved in Raf induced mammary carcinogenesis leads to several testable hypotheses. While it is possible the Raf-Caax constructs create a target for immuno-suppression; we see no histological evidence of immune cell infiltration. Constitutive membrane bound Raf kinase activity may cause apoptosis or differentiation in MECs. Another hypothesis is that lipid modifications on the c-terminus of Raf inhibit signaling or localization to important downstream targets. There are a few sub-categories to the inhibited signalling hypothesis. First, necessary targets within the cell could be unreachable from the plasma membrane (i.e. nuclear or perinuclear targets). Second, lipid modifications could allosterically inhibit interaction with target or scaffolding proteins. Finally, c-terminus attachment could keep the Raf kinase domain too close to the membrane, or improperly oriented for signaling. The serine at amino acid 621, within the kinase domain of Raf, is necessary for kinase activity; this is located just 27 amino acids from the c-terminus of Raf, and less than 45 amino acids from Raf-Caax lipid modifications. In contrast, the Ras binding domain of Raf is more than 500 amino acids from the kinase domain, and the lipid modification on the n-terminus of v-Raf is more than 600 amino acids away. The orientation of Raf-Caax on the membrane may keep its kinase activity too close to the membrane to reach critical targets. Expression of Neu-Raf, n-terminal lipid modified Raf and  $\Delta$ -Raf, Raf-Caax and  $\Delta$ -Raf-Caax with a long glycine linkers between the fusions, and possibly some co-expressed proteins would distinguish these possibilities.
- 8) Lipid modification on the c-terminus of  $\Delta$ -Raf prevents mammary carcinoma induction.
  - Further understanding of the mechanistic details involved in Raf induced mammary carcinogenesis is likely to provide novel targets for the prevention and treatment of human cancer.

## Affinity with Raf is sufficient for Ras to efficiently induce rat mammary carcinomas

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The role of three major Ras downstream effector pathways in the induction of mammary cancer was studied using an *in situ* mammary ductal gene delivery model. Replication-defective retroviral vectors were used to infect endogenous rat mammary epithelial cells with three individual Ras effector loop mutants, each of which transduces its signal through a different Ras effector pathway (Raf, PI3K or RalGDS). Several groups have used Ras effector loop mutants in cultured cells, clearly characterizing the signaling specificity of each over a wide range of cell lines and conditions. Each of the three Ras effector loop mutations impairs Ras for neoplastic transformation of immortal cell lines in culture. In contrast, when evaluated *in vivo* by infecting endogenous rat mammary epithelial cells *in situ* with retroviral vectors, we find that codon 12 mutant activated V12-Ras and all three V12-Ras effector loop mutants individually induce mammary carcinomas. Most notably, a Ras effector loop mutant that lacks affinity with PI3K and RalGDS but retains affinity with Raf (E38-V12-Ras) is relatively similar in potency to V12-Ras for mammary carcinoma induction. Two other Ras effector loop mutants, each lacking affinity with Raf, one retaining affinity with PI3K (C40-V12-Ras), the other with RalGDS (G37-V12-Ras), resulted in much longer tumor latency than E38-V12-Ras and V12-Ras and a reduced carcinoma frequency. Tumor latencies for V12-Ras, E38-V12-Ras, C40-V12-Ras and G37-V12-Ras were 4, 4, 11 and 12 weeks, respectively. We conclude that the Ras–Raf pathway can function independently of the Ras–PI3K and Ras–RalGDS pathways for rapid induction of rat mammary carcinomas, while Ras–PI3K and Ras–RalGDS pathways may also individually induce mammary carcinomas following a long latency.

### Introduction

Activation of Ras is one of the most common molecular events in the etiology of human cancer. This makes Ras an important and heavily researched target for the prevention and treatment of cancer. However, Ras is also involved as a master switch in many normal physiologic functions, increasing the likelihood that inhibiting Ras will lead to systemic toxicity. Cell culture models and biochemical studies have shown that activated Ras

proteins interact directly with several effector proteins, which then induce cellular responses. Targeting the Ras effector pathway(s) that are key to carcinogenesis may minimize drug toxicity, improving the therapeutic index. For these reasons it is important to determine which Ras effector(s) are critical to carcinogenesis. Cell culture models indicate that a minimum of three Ras effector pathways make contributions to carcinogenesis. *In vitro* studies with Ras effector loop mutants are extended here to an *in vivo* epithelial cell model, which more accurately emulates the human disease. In this study we investigate which Ras downstream effectors are important for neoplastic transformation of endogenous rat mammary epithelial cells *in situ*. These cells have never been exposed to the mutating effects of cell culture, or to selection for the low frequency immortal cells from which cell lines historically proceed. Our data are consistent with data from cell lines, suggesting that at least three different Ras effectors contribute to neoplastic transformation. However, induction of rat mammary carcinomas by expression of Ras effector loop mutants *in vivo* is more potent than expected based on cell culture models.

The Ras genes were some of the earliest characterized oncogenes (1,2). Substantial *in vitro* data have allowed the elucidation of mechanisms of transformation in culture (3–6), and many of these findings also apply when cell lines transformed by Ras effector loop mutants in culture are transferred to *in vivo* environments (4,7,8). However, mechanistic *in vivo* data from endogenous epithelial cells, regarding Ras-induced carcinogenesis, has been unavailable. Activation of Ras is a necessary intermediate for signal propagation from most growth factor receptors (9,12), yet several other extracellular stimuli also lead to Ras activation (13–16). Ras activity also has several cellular effects, including mitosis, differentiation (17–19) and apoptosis (20–22). How Ras signaling activity is regulated to create different cellular responses is unclear. Several cellular proteins have been proposed as Ras downstream effectors. One-way Ras activity could lead to different effects is by modulating activation of different downstream effectors.

Normal Ras proteins cycle between active and inactive states. Various stimuli activate Ras by facilitating its interaction with a Ras guanine nucleotide dissociation stimulator (RasGDS), stimulating release of GDP, which allows binding to GTP. Binding to GTP alters the conformation of the Ras effector loop, greatly increasing its affinity with effector proteins [including Raf (23–27), phosphotyrosine-3-kinase (PI3K) (28,29) and RalGDS (30–32)]. Ras GTPase activating protein (Ras GAP) is also an effector (33–38), but it is best characterized as a down-regulator of Ras (39,40). Interaction with Ras GAP increases the intrinsic GTPase activity of Ras by several orders of magnitude, resulting in hydrolysis of bound GTP to GDP, deactivating Ras. Most coding mutations in codon 12 (i.e. V12, E12, etc.) of Ras prevent GTP hydrolysis, resulting in constitutively active Ras.

**Abbreviations:** PI3K, phosphotyrosine-3-kinase; Ras GAP, Ras GTPase activating protein; Ras GDS, Ras guanine nucleotide dissociation stimulator.

With the discovery of many effector proteins, the question of which effector(s) elicit the oncogenic effect of Ras is important. Several researchers have probed this question with Ras effector loop mutants, characterizing them well *in vitro* and in several cell lines (3–8). Mutations in the effector loop of Ras have been shown to greatly reduce the affinity of Ras with its effector proteins. Some of these Ras effector loop mutants are selective, greatly reducing affinity with most effectors yet retaining a significant affinity with select effectors. For instance, mutation from glu to gly in codon 37 (G37-V12-Ras) greatly reduces affinity with Raf and PI3K but retains affinity with RalGDS. In contrast, mutation from asp to glu in codon 38 (E38-V12-Ras) retains affinity with Raf but lacks affinity with RalGDS and PI3K. Complementing these, mutation from tyr to cys in codon 40 (C40-V12-Ras) retains affinity with PI3K but not with RalGDS or Raf. These three Ras effector loop mutants retain affinity with RasGAP, but still allow investigation of the separate contributions that Raf, PI3K and RalGDS make to neoplastic transformation induced by Ras. Multiple groups have characterized Ras effector loop mutants in cultured cells, clearly demonstrating the signaling specificity of each. The importance of synergy between multiple Ras effectors for efficient transformation of cultured cells is a common theme in several studies.

Mutations in the effector loop of Ras substantially attenuate transformation of cultured NIH 3T3 fibroblasts (3,4). However, any pair of Ras effector loop mutants signaling through different effectors synergistically transform 3T3 cell lines (3,4). This suggests that synergy between multiple Ras effectors is necessary for efficient neoplastic transformation. However, NIH 3T3 cell lines transformed by expressing Ras effector loop mutants individually form tumors when injected into nude mice (4). Fibroblasts expressing V12-G37-Ras or V12-C40-Ras take twice as long to form tumors, thus lack of affinity with Raf can reduce the tumor growth rate from transplanted fibroblasts in nude mice (4). Furthermore, subcutaneous tumors from 3T3s expressing V12-G37-Ras or V12-C40-Ras are deficient for metastasis to the lung in nude mice (7). In contrast, others have found that 3T3 cells transformed by V12-G37-Ras (but not C40-V12-Ras) efficiently metastasize to the lung from tail vein injection, although this also seems partially dependent on Erk (the primary effector of Raf-MEK-Erk signal transduction) since it can be inhibited by PAC1 (8). In fibroblasts, individual Ras effector loop mutants have diminished transformation efficiency in comparison with activated Ras, but Raf appears to be a more potent oncogenic effector than PI3K or RalGDS.

Approximately 85% of human tumors originate from epithelial cells, so it is important to test Ras effector loop mutants in epithelial cells. Lack of an appropriate extracellular matrix frequently induces apoptosis in normal epithelial cells; this suspension-induced apoptosis is referred to as anoikis. Immortalized RIE-1 rat intestinal epithelial cell lines or ROSE 199 rat ovarian surface epithelial cell lines are resistant to anoikis when expressing V12-Ras, but are not resistant to anoikis when expressing individual Ras effector loop mutants (5). Anoikis resistance in these cells seems to require Raf activity, but Raf activation is not sufficient for resistance (5). In contrast, the MDCK epithelial cell line is resistant to anoikis when expressing V12-Ras or V12-C40-Ras, suggesting PI3K activity is sufficient to protect MDCK epithelial cells from anoikis (6). Contrasting this is increased *in vitro* invasiveness induced by G37-V12-Ras in 3T3 fibroblast, MCF10A immortalized breast

epithelial cells, and NMuNg mammary cancer epithelial cells (8). As with fibroblasts, epithelial cell lines in culture generally tend to require synergy between multiple effectors. However, unlike cultured fibroblasts where none of these individual effectors seems necessary or sufficient for efficient transformation, in cultured epithelial cells individual pathways appear to be necessary and occasionally sufficient. The next step for extending the data is to evaluate Ras effector loop mutants *in vivo*, with endogenous epithelial cells.

Delivery of Ras effector loop mutants into endogenous epithelial cells *in situ* using replication-defective retroviral vectors infused into the central mammary duct provides a good *in vivo* model for characterization of Ras in the context of mammary carcinogenesis (41). This somatic cell approach has advantages over transgenic models. Retroviral infusion into milk ducts anatomically targets expression to individual cells, driving expression with a constitutive retroviral promoter. This is in contrast to mammary transgenic models that rely on physiological targeting with hormonally responsive promoters. The hormonal stimulus that drives mammary targeting promoters, such as WAP or MMTV, can confound the results of Ras-driven transformation studies, since steroid hormones that drive these promoters also modulate the multistage process of mammary carcinogenesis. In addition, integration positional effects have the potential to modulate observations from transgenic rodents. In contrast, each infused mammary gland contains thousands of infected cells with a random distribution of integration sites between cells. Finally, the low rate of infection in this infusion model (<0.5% of total mammary epithelial cells) allows infected cells to be surrounded by normal cells, as is not the case with germ line transgenic models. As cancer is a clonal disease, it is advantageous to model it in a way that altered cells are surrounded by normal cells. Unfortunately this also makes biochemical characterizations of infected cells difficult until after carcinogenic selection has occurred. We have used this retroviral infusion model system to express three Ras effector loop mutants *in vivo* in order to assess the role of Ras-Raf, Ras-PI3K and Ras-RalGDS signal transduction pathways in mammary carcinogenesis. We find that each of these activated Ras effector loop mutant proteins can yield mammary carcinomas from infused glands with varying efficiency.

## Methods

### Vector construction

Ras effector loop mutant cDNAs were kindly provided by Dr Julian Downward (3). From these templates, PCR products were generated for V12-Ras and the three V12-Ras effector loop mutants. The 5' primer (aaaagcgccgcatgacagaat-acaaactgtgg) contains a *NotI* site, Kozac consensus sequence, and sequence complementing the first few codons of *H-Ras*. The 3' primer (aaaactcgagtcaggacagcacactt) contains an *XhoI* site and sequence complementing the area surrounding the *Ras* stop codon. These four PCR products were subcloned into the JR plasmid (41) by standard procedures. After subcloning, the cDNA sequences were confirmed prior to transfection. These constructs are all identical with the exception of the effector loop mutations.

### Retroviral vector preparation and culture conditions

Cell lines were maintained in DMEM (prepared with 20% of the recommended bicarbonate) containing 25 mM HEPES, 10% FBS and gentamycin. The ecotropic packaging cell line Psi-CRE was transfected with JR plasmid DNA by a lipofectamine protocol (Life Technologies, Rockville, MD). Ecotropic retrovirus was harvested 2 days later and used with 5 µg/ml polybrene to infect the amphotropic packaging cell line PA317. Infected PA317 cells were selected by 10–12 days growth in 0.4 mg/ml G418 (Life Technologies). Resistant colonies were cloned and expanded. Supernatant from expanded clones was titered for virus on 3T3 cells. High-titer clones for each retroviral

vector were tested for the presence of helper virus. A helper virus-free, high-titer ( $>10^5$  c.f.u./ml) clone for each *Ras* construct was further expanded. Two days preceding virus concentration, the temperature was lowered to 33°C to improve viral stability, thus increasing titer. Virus was pelleted by centrifugation at 34 000 g for 2 h through 20% sucrose. The pellets were resuspended in 1/100 original volume of DMEM, then stored at -80°C. High-titer stocks ( $>1 \times 10^7$  c.f.u./ml) were combined, titered and diluted appropriately for infusions.

#### Retroviral titer and helper virus test

Virus was titered by infecting 3T3 cells with diluted viral stocks using 5 µg/ml polybrene, and then these cells were split 2 days later into media containing 0.4 mg/ml G418 for selection. The number of G418 resistant colonies was counted 10–12 days later and the resulting counts were used to estimate the original undiluted viral stock concentrations. To test for helper virus, fresh media was put on infected G418-selected 3T3 cells and the supernatant was used with polybrene 48 h later to attempt infection of fresh 3T3 cells. G418 selection was added 2 days later and a lack of G418 resistant clones suggests an absence of helper virus.

#### 3T3 transformation assay

Highly contact-inhibited Swiss 3T3 cells were maintained at very low density. At the time of cell-cell contact, 3T3 cells from multiple plates were mixed for uniformity to prepare plates of cells for infection. Two days later, just prior to infection, cells were harvested from several plates to determine the number of cells per plate (~200 000). Previously titered retroviral concentrates were thawed and diluted to equal titers, then serially diluted to  $5 \times 10^5$  and  $2.5 \times 10^4$  G418<sup>r</sup> c.f.u./ml retroviral stocks. For combined viral infections, equal amounts of diluted stocks were mixed prior to infection, maintaining  $5 \times 10^5$  G418<sup>r</sup> c.f.u./ml while diluting each vector 2-fold. Diluted stocks (2, 10, 16 and 60 µl) were added with 5 µg/ml polybrene to infect 3T3 cells. Twelve days later each plate was scored for the number of cell colonies with stacked nuclei.

#### Rat infusion and follow up

Rats were maintained with food and water available *ad libitum*, in temperature- and humidity-controlled facilities on a 12 h light/dark cycle. Virgin female Wistar-Furth (Harlan Sprague-Dawley) rats, 50–60 days of age, were used. Retroviral vectors were infused into the central duct of each of 12 mammary glands for each rat. One microliter of 8 mg/ml polybrene (to improve infection) and 1 µl of 20 mg/ml fast green (for visualizing infusion) were added per 100 µl thawed viral suspension immediately before infusion. Rats were anesthetized and the central duct of each gland was cannulated with a 27-gauge blunt-ended needle and infused with about 15 µl of viral suspension. Rats were palpated weekly for tumors following infusion, with size estimates of palpable tumors recorded for each gland. Moribund rats and those that remained at the end of the experiment were necropsied. Tumor samples were fixed in Omni fixative and embedded in paraffin for sectioning and hematoxylin and eosin staining.

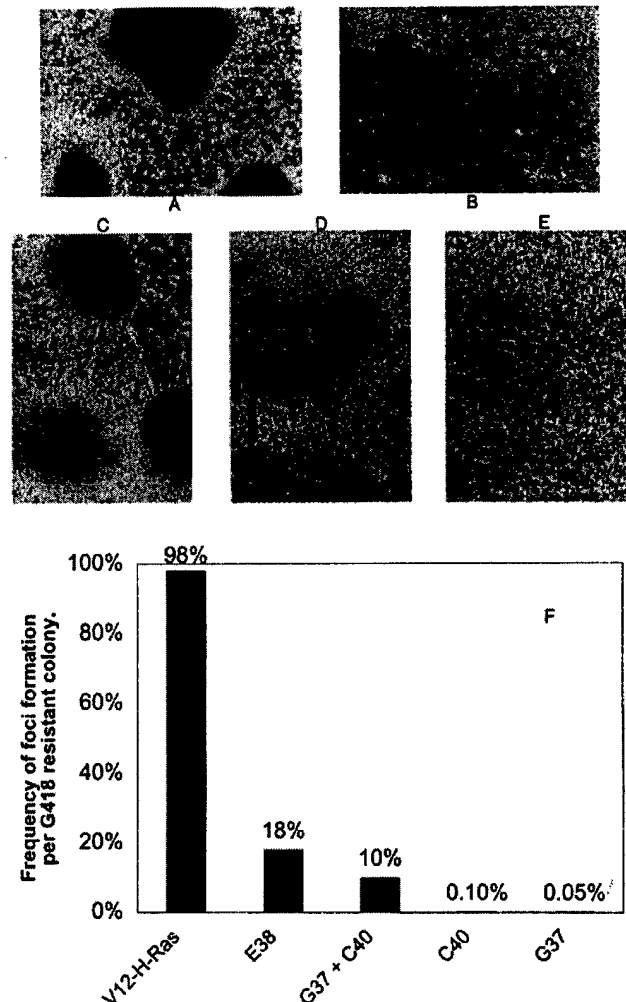
#### Statistical methods

The effect of virus type on the number of positive glands per animal at 7 and 15 weeks was assessed using a generalized linear model assuming Poisson variability. The log-rank test was used to test for an effect of virus type on the time to first tumor. Tests for pairwise differences were performed using similar methods when a significant overall effect of virus was found.

The effect of virus on the growth pattern of the tumors was also assessed. The tumor sizes recorded at the time of each tumor's initial palpation, and the tumor sizes recorded during each of the following 2 weeks, were analyzed without regard for the calendar week in which each tumor first appeared. At each of the three time points, a one-way analysis of variance was used to test for the effect of virus after transforming the tumor sizes to the log scale.

## Results

Cell culture results with retroviral vectors are consistent with published results (3,4) from plasmid transfection experiments: all three individual mutations in the *Ras* effector loop attenuate *Ras* transformation of cultured 3T3 fibroblasts. We find that neoplastic transformation is induced with nearly 100% penetrance in highly contact-inhibited Swiss 3T3 cells by a single retroviral integration expressing V12-Ras (Figure 1F). Foci from V12-Ras infected 3T3 cells became visible to the naked eye within 8 days after infection (confirmed through phase-contrast microscopy) and had frequently divided into foci clusters by day 12 post-infection (Figure 1A). Retroviral



**Fig. 1.** Highly contact-inhibited Swiss 3T3 cells infected with retroviral vectors expressing *Ras* effector loop mutants and the resulting transformation frequencies. Twelve days after infection individual cells expressing V12-Ras (A) generate large clusters of foci, while non-infected control cells (B) stop dividing after reaching confluence. Cells infected with E38-V12-Ras (C) also form foci clusters within 12 days at an attenuated frequency (F). Cells infected with C40-V12-Ras (D) or with G37-V12-Ras (E) did not form clusters of foci but contact inhibition was clearly reduced and cell stacking occurred occasionally (F). For individual vectors, the frequency of transformation (F) is the mean number of foci with stacking nuclei, divided by the mean number of G418 resistant colonies induced by that vector. The numerator for calculating the C40-V12-Ras and G37-V12-Ras co-infection transformation rate is the mean number of foci induced minus the mean number of foci induced by the vectors individually. The denominator for this calculation is itself calculated assuming a Poisson distribution for co-infection, based on the mean number of G418 resistant colonies induced by the vectors individually.

infection with E38-V12-Ras resulted in 3T3 transformation at one-fifth the frequency of V12-Ras (Figure 1C and F), which is more frequent than expected based on plasmid transfection experiments (3,4). The rate of foci formation induced by C40-V12-Ras and G37-V12-Ras expression (0.10 and 0.05% of infected cells, respectively, Figure 1D–F) is comparable with plasmid transfection experiments (3,4). While C40-V12-Ras and G37-V12-Ras rarely induced foci, they did reduce contact inhibition increasing terminal cellular density, and at higher MOIs shortened the time to confluence.

Any pair of the three V12-Ras effector loop mutants is

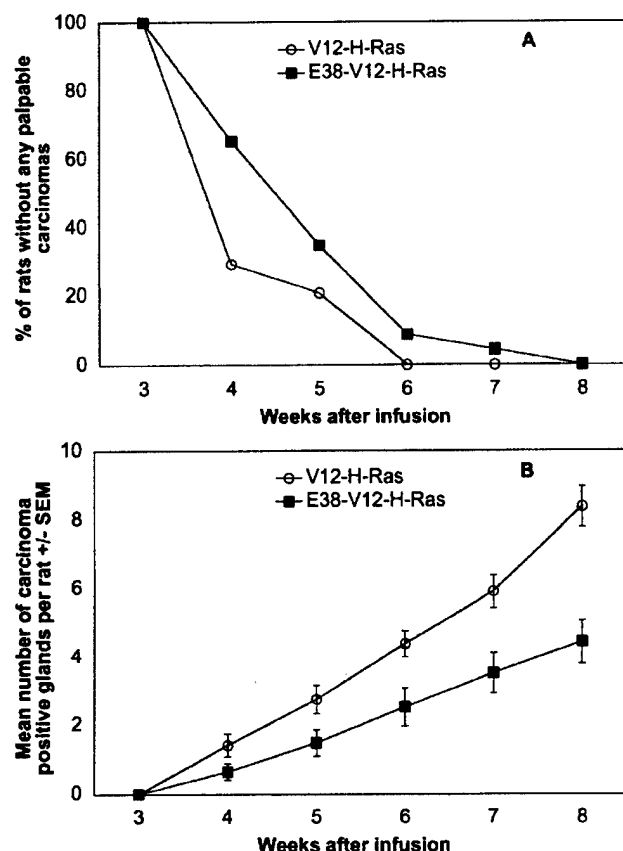


Fig. 2. Latency and frequency of mammary tumor induction from *in situ* expression of V12-Ras and E38-V12-Ras. (A) Latency to palpable carcinoma induction by E38-V12-Ras is not significantly different from V12-Ras carcinoma induction ( $P = 0.0544$ ). (B) Mean frequency, per rat of palpable carcinoma induction by E38-V12-Ras is significantly less than V12-Ras carcinoma induction ( $P < 0.0001$ ). For each vector, all 12 mammary glands of each rat were infused with  $15 \mu\text{l}$  of a standard titer of retroviral suspension ( $1.6 \times 10^7 \pm 0.3 \times 10^7$  c.f.u./ml). E38-V12-Ras was infused into 23 rats, a total of 276 glands. V12-Ras was infused into 24 rats, a total of 288 glands. Rats were palpated weekly. Location and diameter estimates of all palpable mammary tumors  $>3$  mm were recorded.

synergistic for fibroblast transformation (3,4). Synergy between C40-V12-Ras and G37-V12-Ras is quantifiable if we assume Poisson distribution for co-infection. Ten percent of Swiss 3T3 cells co-infected with C40-V12-Ras and G37-V12-Ras form foci (Figure 1F) (the number of foci expected from individual infections was subtracted from the total foci count to estimate the number of foci resulting from co-infection). Transformation from C40-V12-Ras and G37-V12-Ras co-infection is 100 times more frequent than either individually. Individual infections greatly outnumber co-infections, so the high frequency of transformation by E38-V12-Ras infection prevented quantification of E38-V12-Ras synergy with C40-V12-Ras or G37-V12-Ras.

Somewhat like our cell culture results, each of the described Ras effector loop mutants is individually capable of inducing *in situ* rat mammary gland carcinogenesis (Figures 2 and 3). Most notably, the latency of tumor formation resulting from expression of E38-V12-Ras is not significantly different from V12-Ras tumorigenesis ( $P = 0.0544$ ). With viral titers of  $1.6 \times 10^7 \pm 0.3 \times 10^7$  c.f.u./ml, more than half of the rats develop a palpable tumor by the fourth or fifth weeks after

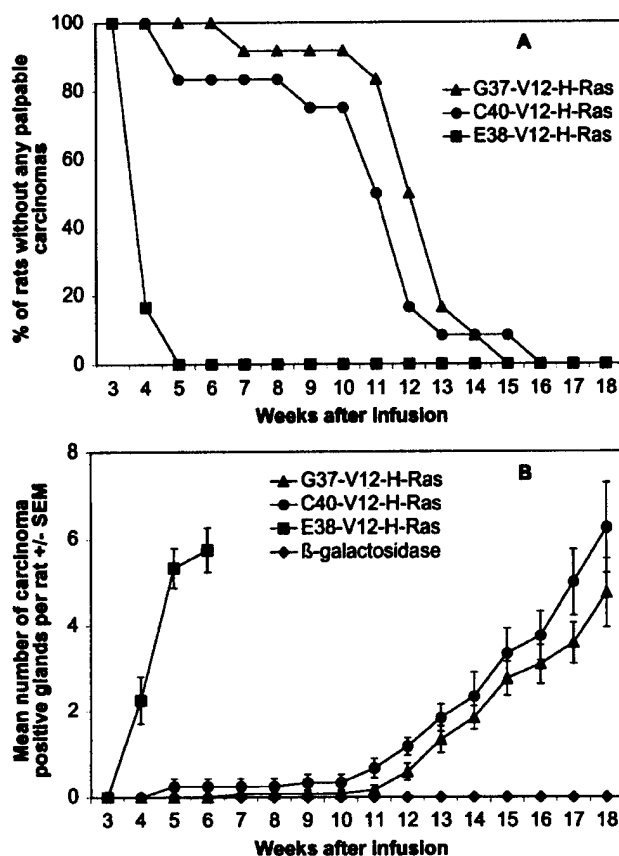


Fig. 3. Latency and frequency of mammary tumor induction from *in situ* expression of G37-V12-Ras, C40-V12-Ras, and E38-V12-Ras. (A) Latency to palpable carcinoma induction by E38-V12-Ras is significantly less ( $P < 0.0001$ ) than for C40-V12-Ras or for G37-V12-Ras. Latency to palpable carcinoma induction by C40-V12-Ras is not significantly different ( $P = 0.2547$ ) from G37-V12-Ras. (B) Mean frequency per rat of palpable carcinoma induction by E38-V12-Ras is significantly greater ( $P < 0.0001$ ) than C40-V12-Ras or G37-V12-Ras. There is a small but significant ( $P = 0.0265$ ) difference between the mean frequency per rat of carcinoma induction by C40-V12-Ras and by G37-V12-Ras.  $\beta$ -galactosidase vector does not induce mammary carcinomas. For each vector 12 rats were infused with  $15 \mu\text{l}$  of high titer retroviral suspension ( $5 \times 10^7 \pm 1.1 \times 10^7$ ,  $6 \times 10^7 \pm 0.3 \times 10^7$  and  $7 \times 10^7 \pm 2 \times 10^7$  c.f.u./ml for G37-V12-Ras, C40-V12-Ras and E38-V12-Ras, respectively) into each of 12 glands, 144 glands with each vector. Location and diameter estimates of all palpable mammary tumors  $>3$  mm were recorded weekly.

infusion of V12-Ras or E38-V12-Ras vectors respectively (Figure 2A). In contrast, the frequency of tumor formation following infusion with E38-V12-Ras is ~55% of that obtained with V12-Ras ( $P < 0.0001$ ) (Figure 2B).

Even at high titers ( $>5 \times 10^7$  c.f.u./ml), it takes 11–12 weeks after infusion with either C40-V12-Ras or G37-V12-Ras before 50% of rats develop a single palpable tumor (Figure 3A). At a similar titer,  $>80\%$  of rats infused with E38-V12-Ras have tumor(s) by the fourth week after infusion (Figure 3A). With C40-V12-Ras or G37-V12-Ras, the appearance of additional palpable tumors is delayed and distributed over a longer time period following detection of the first palpable tumor (Figure 3B). Expression of  $\beta$ -galactosidase from the same retroviral vector backbone does not cause mammary tumors (41). Negative control rats infused with a  $\beta$ -galactosidase expression vector, were followed for over 18 months, with no detectable tumors (Figure 3B, and data not shown). Carcino-

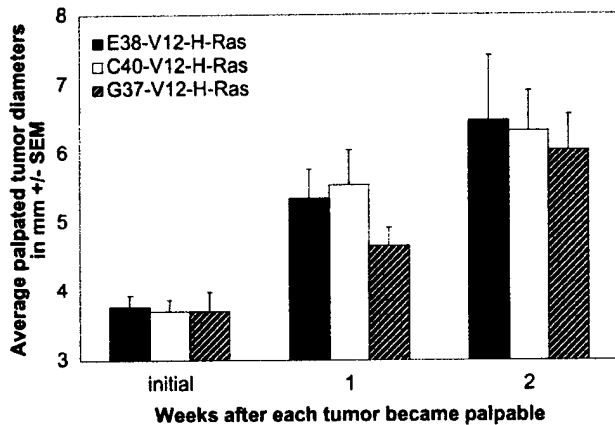


Fig. 4. Mean palpated diameters of mammary carcinomas induced by *in situ* expression of E38-V12-Ras, C40-V12-Ras and G37-V12-Ras effector loop mutants over the first 2 weeks following detection. The tumor diameters recorded at the time of each tumor's initial palpation, and the tumor diameters recorded during each of the following 2 weeks, were analyzed without regard for the calendar week in which each tumor first appeared. Mean diameter of carcinomas at their first recorded palpation is ~3.7 mm, regardless of the Ras vector infused (no significant difference,  $P = 0.8607$ ). There is also no significant difference in the mean diameter estimates of carcinomas from different Ras effector loop mutants the first ( $P = 0.6504$ ) and second ( $P = 0.9686$ ) weeks after initially palpable. All 144 glands of 12 rats were infused for each vector. Location and diameter estimates to the nearest mm of all palpable mammary tumors >3 mm were recorded weekly. Initial palpation diameter means include 62, 54 and 44 carcinomas induced by E38-V12-Ras, C40-V12-Ras and G37-V12-Ras, respectively.

genesis from either of two Ras effector loop mutants that lack affinity with Raf, have long latencies in comparison with those from E38-V12-Ras, yet once initially palpable, individual tumor growth rates are comparable for all three Ras effector loop mutants (Figure 4). Finally an average of five carcinomas from each effector loop mutant group were re-sequenced. No back mutations or other mutations were detected within the effector loops of these carcinomas (data not shown).

Histopathological evaluation reveals that the tumors generated by each vector are exclusively mammary carcinomas, with no consistently discernible histological differences between V12-Ras and V12-Ras effector loop mutant-carrying carcinomas. The great majority of these carcinomas were diagnosed as papillary in nature (Figure 5); however, several cribriform carcinomas were also noted.

## Discussion

Ras effects are signaled through several pathways including Raf, PI3K and RalGDS. In anticipation of investigating the individual capability of V12-Ras effector loop mutants to induce cancer *in vivo* using our retroviral mammary model, we first confirmed that our 3T3 transformation results from retroviral infection, are consistent with the published data using plasmid transfection (3). Transformation of cultured 3T3 fibroblasts was attenuated by all three V12-Ras effector loop mutants. The very low frequency of 3T3 transformation induced by C40-V12-Ras and G37-V12-Ras, when compared with V12-Ras is in agreement with the published data using plasmid transfection.

The low frequency of 3T3 transformation by C40-V12-Ras and G37-V12-Ras individually allows a rough quantification of the synergy between the two. It is important to note that the 100-fold increase is a high estimate. While care was taken

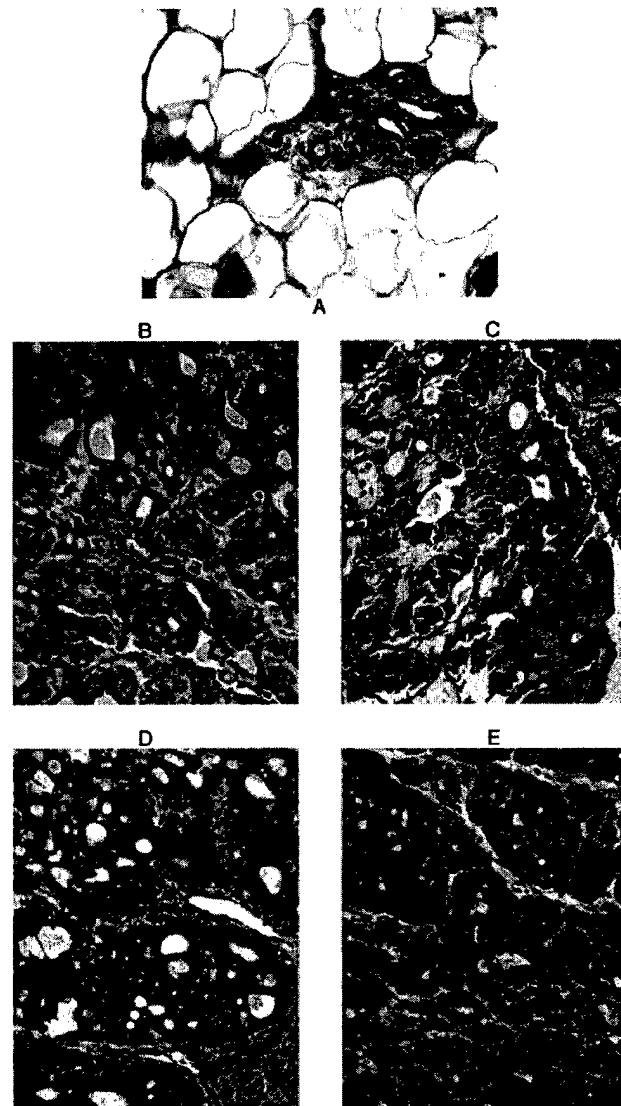


Fig. 5. Rat mammary gland and mammary carcinomas induced by Ras effector loop mutants. In the normal gland (A), adipose cells surround mammary epithelial cells. Mammary carcinomas produced by V12-Ras (B), E38-V12-Ras (C), C40-V12-Ras (D) and G37-V12-Ras (E) have similar morphological characteristics. Tissues were fixed in formalin, embedded in paraffin for sectioning then stained with hematoxylin and eosin. Mammary gland was spread out flat on a slide prior to fixing and sectioned flat.

to assure thorough mixing of vectors in the infecting media, ideal Poisson distribution is unattainable. Less than ideal distribution would increase the number of co-infections and presumably foci, causing a high estimate of transformation frequency induced by co-infection. The rough synergy estimate also assumes the synergistic activities must be contained within a given cell. If paracrine synergy induced extra foci from singly infected cells, this would also contribute to a high estimate of synergy induced by co-infection.

The frequency of neoplastic transformation induced in Swiss 3T3 cells by retroviral infection with E38-V12-Ras is higher than expected based on published plasmid transfection experiments (3,4). Published plasmid transfection experiments with 3T3 cells rely on S35-V12-Ras for Raf specific signaling. S35-V12-Ras has been shown to retain some residual affinity for RalGDS, and also retains less residual affinity for Raf than

E38-V12-Ras (3). These were the reasons for our choosing E38-V12-Ras over S35-V12-Ras, and may also account for the increased frequency of foci formation in 3T3 cells. The low passage highly contact-inhibited Swiss 3T3 used here could also be more sensitive to transformation by Raf than the 3T3 strains that were used in plasmid transfection experiments (3,4). However, we have found that the rate of transformation from infection with N-terminal truncated Raf ( $\Delta$ -Raf, manuscript in preparation) conforms very well to published results from plasmid transfection experiments (3). While the frequency of neoplastic transformation in 3T3 cells from E38-V12-Ras was higher than expected, it was nonetheless clearly attenuated. In addition to having only one-fifth the penetrance of V12-Ras in 3T3 cells, foci induced by E38-V12-Ras were less prone to becoming clusters of foci.

In partial contrast to results in culture, V12-Ras and all three V12-Ras effector loop mutants efficiently induce carcinomas in the rat mammary gland. Most notably, mammary carcinoma latency and morphology from E38-V12-Ras are not significantly different from those induced by V12-Ras. The multiplicity of carcinoma induction by E38-V12-Ras is about half as frequent as that induced by V12-Ras. As E38-V12-Ras retains <40% of its affinity with Raf (3), one might expect attenuated transformation, even if Raf were the only oncogenic effector of Ras. However, Ras effectors compete for the effector loop of V12-Ras, so reduced competitive inhibition with PI3K and RalGDS for the E38-V12-Ras effector loop may compensate for reduced affinity with Raf. Furthermore, competitive inhibition between E38-V12-Ras and endogenous Ras for the down-regulator RasGAP could increase endogenous Ras activity, providing low level PI3K and/or RalGDS activity, with which high Raf activity may act synergistically. Regardless of competitive inhibition possibilities, our *in vivo* E38-V12-Ras data suggest that Raf is the primary oncogenic effector of Ras and that PI3K and RalGDS make at most a limited contribution to Ras transformation of mammary epithelial cells *in situ*. We have recently confirmed the ability of Raf activation to induce rat mammary carcinomas with *in situ* expression of  $\Delta$ -Raf (manuscript in preparation). Efficient carcinoma induction by E38-V12-Ras suggest major anticancer effects could be obtained by targeting the Ras-Raf pathway, and that targeting PI3K and RalGDS without Raf is unlikely to treat activated Ras-containing tumors effectively.

Tumorigenesis from G37-V12-Ras or C40-V12-Ras has much greater latency than E38-V12-Ras and V12-Ras, emphasizing the importance of Raf for efficient and rapid transformation. There are several interpretations of what these results suggest regarding the role of PI3K and RalGDS in carcinogenesis. This may reflect additional independent oncogenic contributions from PI3K and RalGDS, which are unnecessary in the presence of sufficient Raf signal. However, as seen in this model with wt-H-Ras, deregulated over-expression itself may play a significant role in delayed transformation (42). Deregulated over-expression could indirectly up-regulate endogenous Ras proteins by sequestering RasGAP, similar to the way dominant-negative Ras indirectly down-regulates endogenous Ras proteins by sequestering RasGDS. Endogenous Ras might then provide Raf signaling sufficient for carcinogenesis. PI3K or RalGDS activity may also signal for increased Raf activity, for example through a Ras-PI3K-PKB-Rac-Pak3 pathway (43,44), so tumors from G37-V12-Ras or C40-V12-Ras expression might have high Raf activity. While V12-E38-Ras data suggest that Raf activity can be

sufficient for rat mammary gland tumorigenesis, V12-G37-Ras and V12-C40-Ras data suggest Raf activity may not be necessary following long latency.

In summary, we find that Ras affinity with PI3K and RalGDS is unnecessary for rapid carcinogenesis from *in situ* rat mammary epithelial cells. Either of two Ras effector loop mutants that lack affinities with Raf were substantially impaired for their *in vivo* transformation ability. We conclude that Raf is the primary oncogenic effector of Ras, and Raf activation can initiate rat mammary gland carcinogenesis. Affinity of activated Ras with Raf is necessary and sufficient for rapid mammary gland carcinogenesis, while affinity with PI3K or RalGDS is sufficient for carcinogenesis after long latency. Our *in vivo* mammary epithelial cell data suggest that the Raf pathway may serve as the single most important pathway to target for the prevention and treatment of tumors containing active Ras.

### Acknowledgements

We thank Jill Haag for technical assistance, Stephanie Nelson for help in re-sequencing, and Laurie Shepel for editing the manuscript. National Institutes of Health/ National Cancer Institute grant CA77527 and US Army Medical Material Command pre-doctoral fellowship grant DAMD17-98-1-8357 supported this work.

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Received May 29, 2002; revised August 30, 2002; accepted September 5, 2002